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Cultivable microbial ecology and aromatic profile of “mothers” for Vino cotto wine production

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34 **Cultivable microbial ecology and aromatic profile of “mothers” for Vino cotto wine**
35 **production**

36

37 Noemi Battistelli ^{a,1}, Giorgia Perpetuini ^{a,1}, Andrea Piva ^a, Alessia Pepe ^a, Rossana Sidari ^b, Yves Wache ^{c,d,e},
38 Rosanna Tofalo ^{a,*}

39

40 ^a Faculty of BioScience and Technology for Food, Agriculture and Environment, University of Teramo, Via R.
41 Balzarini, 1, 64100 Teramo, Italy

42 ^b Department of Agraria, Mediterranean University of Reggio Calabria, Loc. Feo di Vito, I-89122 Reggio Calabria,
43 Italy

44 ^c Tropical Fermentation Network, France

45 ^d International Joint Laboratory, Tropical Bioresources & Biotechnology, Univ. Bourgogne Franche-Comté, AgroSup
46 Dijon, PAM UMR A 02.102 and School of

47 Biotechnology and Food Technology, Hanoi University of Science and Technology, Dijon, France

48 ^e School of Food Science and Technology, Faculty of Science and Technology, Thammasat University, 12120, Thailand

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* Corresponding author.

E-mail address: rtofalo@unite.it (R. Tofalo).

¹ These authors equally contributed to this work.

76 A B S T R A C T

77 The aim of the present study was to assess the cultivable microbiota of “mothers” of *Vino cotto*
78 collected from production of different years 1890, 1895, 1920, 1975, 2008. A total of 73 yeasts and
79 81 bacteria were isolated. *Starmerella lactis-condensi*, *Starmerella bacillaris*, *Hanseniaspora*
80 *uvarum*, *Saccharomyces cerevisiae*, *Hanseniaspora guillermondi* and *Metschnikowia pulcherrima*
81 were identified. Bacteria isolates belonged to lactic acid bacteria (*Lactiplantibacillus plantarum* and
82 *Pediococcus pentosaceus*) and acetic acid bacteria (*Gluconobacter oxydans*). Remarkable
83 biodiversity was observed for *Starm. bacillaris*, as well as *L. plantarum* and *G. oxydans*. Organic
84 acids and volatile compounds were also determined. Malic and succinic acids were the main ones
85 with values ranging from 8.49 g/L to 11.76 g/L and from 4.15 g/L to 7.73 g/L respectively, while
86 citric acid was present at low concentrations (<0.2 g/L) in all samples. Esters and higher alcohols
87 were the main volatile compounds detected followed by alkanes. This study permits to better
88 understand the microbial communities associated to this product and could be considered a starting
89 point for the definition of tailored starter cultures to improve the quality of *Vino cotto* preserving its
90 typical traits.

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104 *Keywords:*

105 Osmotolerant yeast

106 Lactic acid bacteria

107 Acetic acid bacteria

108 Aroma compounds

109 *Vino cotto*

110 **1. Introduction**

111 Vino cotto (cooked wine) is a typical sweet wine produced in Abruzzo and Marche regions. It was
112 inserted in the national list of traditional food products for Marche and Abruzzo regions in 2000 and
113 2003, respectively, and can be marketed as traditional agrifood product (Repubblica Italiana 2000;
114 2003). It is produced according to traditional procedures using different grape cultivars including
115 Trebbiano, Passerina, Montonico, Moscato, Montepulciano and Sangiovese. The must is heated and
116 concentrated to 30–70% in copper boilers. To start the alcoholic fermentation fresh must can be
117 added so the indigenous yeasts can drive the fermentation process which proceeds very slowly for
118 more than a month at room temperature because of the high concentration of sugar and other
119 naturally occurring substances such as acids, polyphenols, metal ions, and the formation of Maillard
120 reaction products (Piva et al., 2008). Once the fermentation ends Vino cotto is transferred in
121 wooden barrels. In the bottom of barrels, the must/wine which settles over the years and gives the
122 characteristic aroma and flavour of Vino cotto is present (Tofalo et al., 2009). This mixture is called
123 by local producers “mother” of Vino cotto. Therefore, each barrel is a unique niche where a peculiar
124 microbiota can develop. Very few studies have been carried out to study the microorganisms hosted
125 by “mother” of Vino cotto and Vino cotto wine. It represents a very stressing environment since
126 microorganisms are exposed to osmotic stress which cause the loss of intracellular water and the
127 collapse of cytoskeleton (Hohmann, 2002). In general, osmotolerant yeasts could grow facing the
128 stressing conditions since they are able to retain the ability to synthesize glycerol as a compatible
129 solute or osmoregulator, and some yeasts even have active glycerol uptake pumps (Hohmann,
130 2002). In a previous study Tofalo et al. (2009) isolated, identified and characterized the
131 predominant indigenous yeast species during Vino cotto production. Only four species were
132 identified: *Saccharomyces cerevisiae*, *Candida apicola*, *Starmerella bacillaris* (syn. *Candida*
133 *zemplanina*) and *Zygosaccharomyces bailii*. All the species showed osmotolerant traits being able to
134 develop in presence of high concentration of glucose in a strain dependent way.

135 No data are available concerning bacteria. However, some bacterial group can also develop. In
136 particular, lactic acid bacteria (LAB) and acetic acid bacteria (AAB).

137 LAB are able to face different stress conditions since they Harbour specific genes and they have
138 evolved adaptive networks such as the so called Global Regulatory Systems. It controls the
139 simultaneous expression of a large number of genes in response to a variety of environmental stress
140 factors (Spano & Massa, 2006). Moreover, they were isolated in “Shanxi aged vinegar”, a
141 traditional Chinese vinegar (Wu, Ma, Zhang, & Chen, 2012).

142 AAB are found on stressing substrates containing sugars and/or alcohol, such as fruit juice, wine,
143 cider, beer, and vinegar (Mas, Torija, García-Parrilla, & Troncoso, 2014; Calabrò, Fazzino, Sidari,

144 & Zema, 2020) and they are able to survive in Traditional Balsamic Vinegar, another Italian
145 traditional product made with cooked grape must (Solieri, Landi, De Vero, & Giudici, 2006).
146 In light of the above, the aim of the present work was to determine the cultivable microorganisms
147 associated with “mothers” of Vino cotto collected from barrels of different years (1890, 1920, 1926,
148 1980, 2008). Specifically, AAB, LAB and yeasts were enumerated and isolated. Isolates
149 identification was performed as well as their genotypic grouping in order to verify if a core
150 microbiota was conserved over years. Moreover, samples were characterized for organic acids and
151 polyphenols content and volatile profile.

152

153 **2. Materials and methods**

154 *2.1. Samples origin*

155 Five barrels of different years (1890, 1895, 1920, 1975, 2008) containing “mothers” of Vino cotto
156 were analyzed. Samples were named as follow: V1890, V1895, V1920, V1975, V2008. Samples
157 were obtained from a local winery of Abruzzo region, collected in sterile tubes and transported to
158 the microbiological laboratory of the Faculty of BioScience and Technology for Food, Agriculture
159 and Environment (University of Teramo) and analyzed.

160 *2.2. Microbial counts*

161 Samples were diluted in physiological solution (NaCl 0.85% w/v) and serially diluted. Cell
162 suspensions were spread-plated and incubated as follows: yeasts on YPD agar (Yeast Extract 10
163 g/L, Peptone 20 g/L, Dextrose 20 g/L, Agar 18 g/L) and on Wallerstein Laboratory Nutrient Agar
164 (WLN) (Oxoid, Milan, Italy) at 28 °C for 48 h; LAB on DeMan-Rogosa-Sharp Agar (MRS)
165 (Oxoid) with 100 ppm cycloheximide at 30 °C for 48 h in microaerophilic conditions; AAB on
166 GYC medium (Glucose 100 g/L, Yeast Extract 10 g/L, Calcium Carbonate 20 g/L, Agar 1.5 g/L) –
167 a common medium useful to isolate AAB from sources rich in sugar – at 30 °C for 48 h in aerobic
168 conditions (Gullo, Caggia, De Vero, & Giudici, 2006). Cell counts were performed in duplicate.

169 The random colony selection from the highest dilution plates allowed us to collect the most frequent
170 species present in each sample, as reported by Tofalo et al. (2009), and Solieri et al. (2006). Isolates
171 were purified and stored in liquid cultures with glycerol (20% v/v) (Sigma Aldrich, Milan, Italy) at -
172 80 °C. Strains belong to the Culture Collection of the Faculty of BioScience and Technology for
173 Food, Agriculture and Environment (University of Teramo).

174

175 *2.3. Yeasts identification and typing*

176 Yeast cells were grown aerobically in YPD at 28 °C. DNA was extracted according to Aa,
177 Townsend, Adams, Nielsen, and Taylor (2006). The 5.8S internal transcribed spacer (ITS) rRNA

178 region was amplified in a Bio-Rad thermocycler (MyCycler, Bio-Rad Laboratories, Milan, Italy)
179 using primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5'
180 TCCTCCGCTTATTGATATGC 3'). The PCR product was digested with the restriction enzymes
181 CfoI, HaeIII and HinfI as previously described (Esteve-Zarzoso, Belloch, Uruburu, & Querol, 1999;
182 Tofalo et al., 2009). In order to have a confirmation of the species, sequencing of the D1/D2
183 domains of 26S rRNA gene was conducted using primers NL1 (5'
184 GCATATCAATAAGCGGAGGAAAAG 3') and NL4 (5' GGTCCGTGTTTCAAGACGG 3')
185 (Kurtzman & Robnett, 1998). The PCR product was purified by ExoSAP-IT (Thermofisher, Milan,
186 Italy) according to manufacturer's instructions and delivered to BMR Genomics (Padua University,
187 Padua, Italy) for sequencing. The obtained sequence were compared to those available in the
188 GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>) and those of the Ribosomal Database
189 Project (<http://rdp.cme.msu.edu/index.jsp>) to determine the closest known relative species on the
190 basis of 26S rRNA gene homology (Altschul et al., 1997).

191 Strains were typed by RAPD-PCR with primer M13 (5' GAGGGTGGCGGTTCT 3') as previously
192 described (Tofalo et al., 2009). Fingerprinting II InformatixTM software program (Bio-Rad) was
193 employed for conversion and normalization of the RAPD-PCR patterns. Similarities among profiles
194 were calculated by clustering the Pearson's r correlation matrix using the Unweighted Pair-Group
195 Method with Average (UPGMA) algorithm.

196

197 2.4. Bacteria identification and typing

198 DNA was extracted using InstaGene Matrix (Bio-Rad) according to manufacturer's instructions.
199 Bacterial isolates were identified by 16S rRNA gene sequencing. PCR reactions were performed as
200 described by Bringel et al. (2005) using Lac16S-for (5' AATGAGAGTTTGATCCTGGCT 3') and
201 Lac16S-rev (5' GAGGTGATCCAGCCGCAGGTT 3') primer set. PCR products were purified and
202 sent to BMR Genomics for sequence analysis. *Lactiplantibacillus plantarum*, *L. pentosus*, and *L.*
203 *paraplantarum* were differentiated by multiplex PCR according to Torriani, Felis, & Dellaglio
204 (2001). Molecular typing was performed by M13 RAPD-PCR as previously described.

205

206 2.5. Organic acids analysis

207 Organic acids of "mothers" of Vino cotto samples were detected by HPLC according to Tašev,
208 Stefova, and Ivanova (2016) and Piva et al. (2008). All chemicals were of analytical reagent grade
209 and supplied by Sigma Aldrich. To separate organic acids from other components, solid phase
210 extraction (SPE) was performed. Samples were diluted 1:10 and filtered, then extracted with SPE
211 using Supelclean LC-18 SPE 500 mg cartridges (Sigma Aldrich). The cartridges were conditioned

212 with 2 mL methanol and 2 mL water. Then, 500 μ L of samples were loaded on the cartridges.
213 Elution was performed with two portions (500 μ L each) of buffered water at pH 2.1. Finally, 10 μ L
214 of the eluate was injected into the HPLC system for analysis. Organic acids (citric acid, tartaric
215 acid, malic acid, succinic acid, lactic acid and acetic acid – Sigma Aldrich) were used to create the
216 standard curve. The detector was an HPLC 200 series (Perkin Elmer, Monza, Italy) connected to a
217 UV VIS detector at 210 nm. ROA Organic Acid H + column (Phenomenex, Bologna, Italy) was
218 used. All determinations were performed isocratically with a flow rate of 0.7 mL/min at 65 °C using
219 H₂SO₄ solution 0.009 N as mobile phase. Analyses were performed in triplicate.

220

221 *2.6. Volatile compounds*

222 Volatile compounds were analyzed using gas chromatography/mass spectrometry (GC–MS) as
223 described by Tofalo et al. (2016). Analyses were performed using a Clarus SQ8S
224 chromatography/mass (GC–MS) spectrometry (Perkin Elmer, Boston, MA). The column used was a
225 capillary GC column (30 m \times 0.25 mm i.d. 0.25 μ m film thickness) coated with polyethyleneglycol
226 (film thickness 1.2 μ m), as stationary phase. A carboxen–polydimethylsiloxane-coated fiber (85
227 μ m) was used (Sigma-Aldrich, St. Louis, MO, USA). Equilibration and adsorption steps were
228 performed stirring the samples for 30 min at 40 °C. The fiber was placed in the injector (T = 250
229 °C) for 15 min and the following program was applied: 50 °C for 2 min; first ramp, 1 °C min to 65
230 °C; second ramp, 10 °C min to 150 °C (10 min hold); third ramp 10 °C min to 200 °C (1 min hold).
231 Volatile compounds were identified comparing mass spectra of compounds with those contained in
232 the available database (NIST version 2005). All determinations were performed in triplicate.

233

234 *2.7. Statistical analysis*

235 Organic acids and volatile compounds results were expressed as mean value \pm standard deviation.
236 The Friedman’s test was used and $p < 0.01$ was used as criterion for statistical significance.
237 Principal component analysis (PCA) based on the main volatile compounds, organic acids and
238 samples was performed using the software XLStat 2014 (Addinsoft, New York, NY, United States).

239

240 **3. Results and discussion**

241 *3.1. Microbial counts*

242 This study evaluated viable microbial communities hosted by “mothers” of Vino cotto of different
243 years. Yeasts were detected in all samples with values ranging from 2.7 Log CFU/mL (V1890) to
244 3.5 Log CFU/mL (V2008) (Fig. 1). These results are similar to other studies on high content sugary
245 foods (i.e. honey and manna), which are recognized as a stressful environment, and allow the

246 growth of osmotolerant microorganisms (Snowdon & Cliver, 1996; Fe'as, Pires, Iglesias, &
247 Estevinho, 2010; Sinacori et al., 2014; Guarcello et al., 2019). Yeast counts increased over time
248 showing the highest values in samples obtained from the newest barrels. LAB showed a similar
249 trend. They were present only in “mothers” of *Vino cotto* collected from the most recent barrels
250 ranging from 2 Log CFU/mL (V1980) to 6.12 Log CFU/mL (V2008). In the other samples they
251 showed values of microbial count less than the limit of detection. An opposite tendency was
252 observed for AAB. In fact, they were detected in the oldest 3 samples with values from 4 Log CFU/
253 mL (V1890) to 2 Log CFU/mL (V1920), while were absent in “mothers” of *Vino cotto* obtained
254 from the newest barrels (V1975, V2008) (Fig. 1).

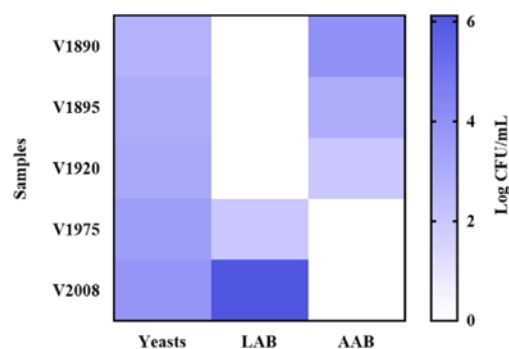
255 Therefore, samples from barrels of V1890, V1895 and V1920 were characterized by the presence of
256 yeasts and AAB, while those of V1975 and V2008 by yeasts and LAB. The different occurrence of
257 microbial groups could influence fermentation kinetics resulting in different organoleptic
258 characteristics which make this product unique and with traits which are different from year to year.
259 Probably, the occurrence of AAB is influenced by oxygen availability, which is higher in the oldest
260 barrels.

261 No data are available concerning the microbial groups characterizing “mothers” of *Vino cotto*.
262 Tofalo et al. (2009) studied the yeast population occurring during the different steps of *Vino cotto*
263 production. At the beginning of fermentation, after the addition of fresh must, yeast counts were 6.3
264 Log CFU/mL, and a maximum number of 8.2 Log CFU/mL was reached during fermentation, while
265 at the late stages of the process the viable cells decreased to 5.1 Log CFU/mL.

266 However, the same yeast species were detected by other authors in similar products obtained by
267 cooked must, such as balsamic vinegars (TBV) (Solieri et al., 2006; Gullo et al., 2006). The
268 presence of AAB is associated to their ability to resist to acid environment (pH 2.5 – 3.5).

269 Moreover, they are able to grow at elevated sugar concentration characterizing this product (Gullo
270 et al., 2006). LAB are able to tolerate different stressing conditions thanks to their genetic, and can
271 promote a soft taste by moderating the irritating sour smell (Chen, Li, Qu, & Chen, 2009).

272



273

274

Fig. 1. Viable count of yeasts, LAB and AAB.

275 3.2. Yeast identification and typing

276 Colonies were selected on WLN and YPD media according to colony shape, colour and surface
277 features. To attribute the species, PCR-RFLP and sequencing of D1/D2 domain of 26S rRNA gene
278 were performed and compared with those available in the EMBL nucleotide sequence database.
279 (Table S1). All the sequences obtained displayed similarity values ranging from 99 to 100%.

280 As regards yeasts, a total of 73 colonies were isolated. *Starmerella lactis-condensi* (30), *Starm.*
281 *bacillaris* (16), *Hanseniaspora uvarum* (13), *Saccharomyces cerevisiae* (7), *Hanseniaspora*
282 *guillermondi* (5) and *Metschnikowia pulcherrima* (2) species were identified (Fig. 2). *Starm.lactis-*
283 *condensi*, which was present in all samples, is a relative of *Starmerella stellata* and *Starm. bacillaris*
284 (syn. *Candida zemplinina*). The yeasts belonging to these species are osmotolerant and able to grow
285 also on 50% w/w glucose, a typical characteristic of *Starmerella* clade (Kurtzman, Fell & Boekhout,
286 2011). Osmotolerant yeasts are characterized by their ability to survive high osmotic pressure in the
287 environment, caused by the presence of sugars or salts. The adaptation to a low water activity (a_w)
288 environment is a species and strain specific trait and it is generally based on modification in plasma
289 membrane composition, activity of various ion transporters or redox metabolism (Hohmann, 2002;
290 Thomè, 2007). *Starm lactis-condensi* is nutritionally specialized and has been previously found in
291 sugar syrups, manna and TBV. Probably, it could be brought on fruits by insects, like bees and
292 wasps, which could be a vector for their spreading (Lievens et al., 2015; Solieri & Giudici, 2008;
293 Guarcello et al., 2019). The occurrence of *Starm. bacillaris* (syn. *C. zemplinina*) in “mothers” of
294 *Vino cotto* is not surprisingly, since Tofalo et al. (2009) found this species throughout all the
295 fermentation process of *Vino cotto*. Its presence is related to its osmotolerance, in fact it grows
296 like or better than *S. cerevisiae* in media with high sugar contents (Tofalo et al., 2009). Several
297 ecology studies reported the occurrence of *Starm. bacillaris* in both white and red wines from
298 different origin as well as in botrytized grape (Masneuf-Pomarede et al., 2015). When it is used in
299 mixed fermentation with *S. cerevisiae*, it produces wine with reduced ethanol content and an
300 increased concentration of glycerol (Englezos et al., 2015). This trait is interesting since the global
301 warming and the evolution of viticulture practices led to grape must with increased sugar content
302 and thus increased potential ethanol content (Englezos et al., 2015). *Starm. bacillaris* presents other
303 interesting features such as the fructophilic character (Magyar & Tóth, 2011; Tofalo et al., 2012;
304 Englezos et al., 2015), and the antifungal activity against *Botrytis cinerea* (Lemos et al., 2016).

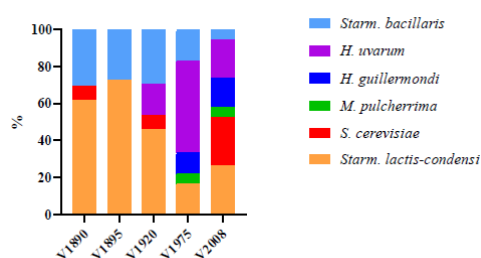
305 The ascomycetous yeast *Metschnikowia* dominates most nectar microbial communities and it is
306 often transferred from insects or birds, from flower to flower or from flower to fruit (Lievens et al.,
307 2015). Sugar composition and concentration is crucial for its development, but this species is able to

308 exploit a diversity of resources efficiently, which help it to survive in nectars (Herrera, Pozo, &
309 Bazaga, 2012). In high sugary foods, such as high sugar grape musts, wines produced with dried or
310 botrytized grapes, honey or manna, non-*Saccharomyces* yeasts are usually dominant (Sinacori et al.,
311 2014; De Filippis et al., 2019). *S. cerevisiae* is not recognized as osmotolerant yeast, but its survival
312 is a strain specific characteristic, and it has also been previously isolated in Vino cotto and TBV
313 (Tofalo et al., 2009; Solieri & Giudici, 2009). In this study, a total of 7 isolates of *S. cerevisiae* have
314 been found in the “mothers” of Vino cotto of the following years V1890, V1920, V2008.

315 *H. uvarum* was isolated from V1920, V1975 and V2008 samples. It is usually predominant in the
316 early stages of wine fermentation or fruit juices, and it can occur also during middle and late phases
317 (Kurtzman et al., 2011). Some strains previously showed fructophilic character and relevant
318 glycerol production (De Benedictis, Bleve, Grieco, Tristezza, & Tufariello, 2011). It has been found
319 in sweet wines such as passito wines (De Filippis et al., 2019) or pulque, an alcoholic beverage
320 from agave (Steinkraus, 2002).

321 Repeatability of RAPD-PCR fingerprints was determined by triplicate loading of independent
322 triplicate reaction mixtures prepared with the same strain and a limited variability in the number and
323 length of the resulting bands. The reproducibility of PCR assays and running conditions was higher
324 than 90%. Banding patterns with a level of similarità higher than 90% was considered as a biotype.
325 UPGMA dendrogram is shown in Fig. 3. RAPD-PCR resulted in a coherent classification at the
326 species level. A single biotype was found for *H. uvarum*, *H. guilliermondi*, *M. pulcherrima*, *Starm.*
327 *lactis-condensi* and *S. cerevisiae* strains, while two biotypes for *Starm bacillaris* strains. These
328 results suggested strain adaptability to this niche along the year of production sampled. This low
329 RAPD-PCR diversity can be explained by the supposed prevalence of a small number of dominant
330 species or “core” strains, selected by the stressful conditions imposed by this peculiar environment,
331 similarly to what happens in other ecological niches (Biolcati, Andrighetto, Bottero, & Dalmaso,
332 2020).

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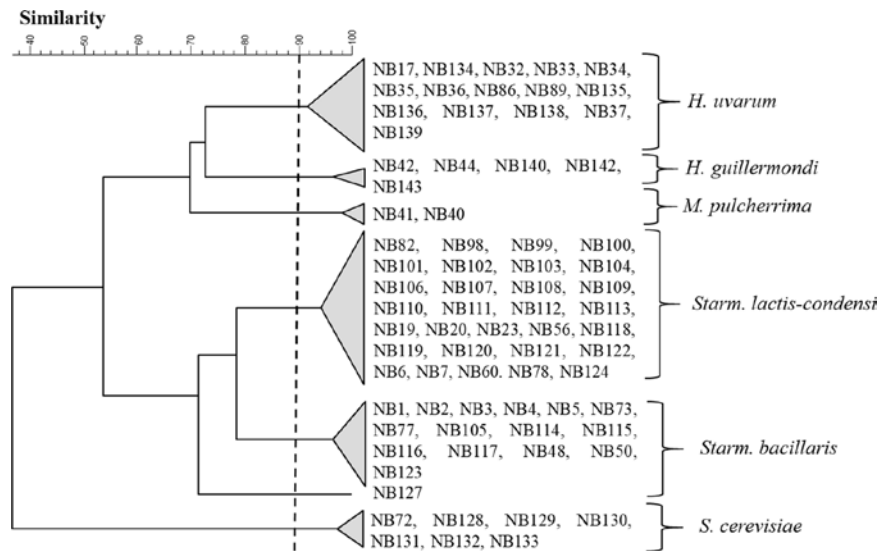
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Fig. 2. Percentage of yeasts species.



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339

340 Fig. 3. RAPD-PCR cluster analysis of yeasts isolates. Unweighted pair group method with arithmetic mean
341 (UPGMA) dendrogram derived from comparison of the RAPD-PCR patterns of the yeast isolates obtained
342 with primer M13.

343

344 3.3. Bacterial identification and typing

345 A total of 81 colonies were isolated and only 3 species were identified (Table S2). In samples V1890, V1895
346 and V1920 only *Gluconobacter oxydans* (36) was found. In samples V1975 and V2008 were isolated LAB
347 belonging to *Pediococcus pentosaceus* (12) and *L. plantarum* (33) species (Fig. 4). The confirmation of *L.*
348 *plantarum* species was conducted after amplification of *recA* gene which resulted in the presence of the band
349 at 318 bp for all the strains.

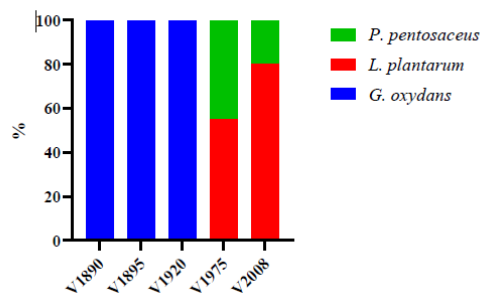
350 Generally, *G. oxydans* can be found in sugary niches such as flowers and fruits (De Muynck et al., 2007) and
351 can be isolated from honey, cider, beer, wine and vinegar. The presence of *G. oxydans* in the “mothers” of
352 Vino cotto is probably related to its metabolic traits: they can obtain energy from sugars efficiently via
353 pentose phosphate pathway and glucose oxidation lead to the production of gluconic acid and can improve
354 the flavour of the final product (Macauley, McNeil, & Harvey, 2001; De Muynck et al., 2007).

355 *Lactiplantibacillus plantarum* and *P. pentosaceus* isolates were found in the two most recent samples, V1975
356 and V2008. LAB are generally highly demanding regarding nutrients, and sugar-rich environments can
357 inhibit their growth. Some fructophilic LAB have been found: they are able to develop in highly sugary
358 niches such as flowers, nectar and fruits, in the digestive tracts of pollinators, or in fermented foods derived
359 from fruits (Endo & Okada, 2008; Endo, Futagawa, & Dicks, 2009). Recently, some evidence about the
360 frucophilic attitude of a *L. plantarum* strain isolated from honeydew have been described (Gustaw, Michalak,
361 Polak-Berecka, & Waśko, 2018). It is likely that the strains found in our samples derived from the grapes
362 and were able to adapt themselves to the osmophilic environment they found, being able to survive.

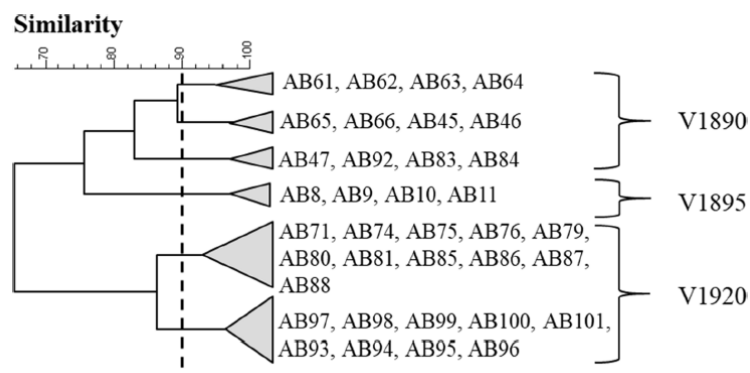
363 *P. pentosaceus* is generally isolated in wine environment (must, alcoholic/malolactic fermentation, aging and
364 conservation). It can grow in wines that are considered microbiologically stable, and its presence does not

365 always lead to spoilage, but can be useful for the malolactic fermentation. New findings have suggested
 366 potential uses for *Pediococcus* spp. in winemaking but have also underlined the necessity to further study the
 367 factors that influence its growth and spoilage potential (for a review see Wade, Strickland, Osborne, &
 368 Edwards, 2019).

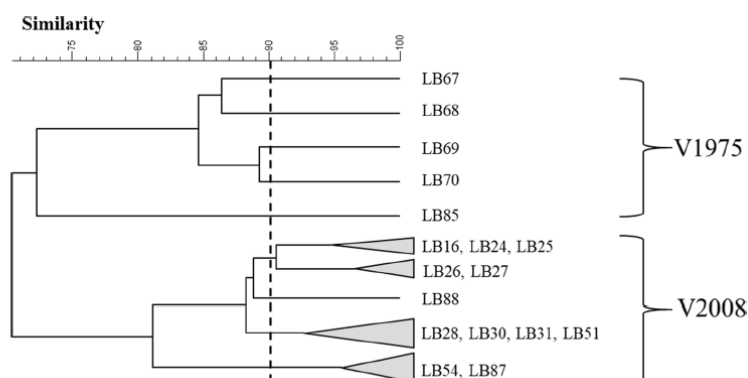
369 All bacteria were processed by RAPD-PCR analysis to differentiate them at strain level. A single biotype
 370 was obtained for *P. pentosaceus* strains (data not shown). On the contrary, 6 biotypes for *G. oxydans* were
 371 detected. A relationship with sample origin was found (Fig. 5). V1890 sample showed 3 different profiles,
 372 V1895 sample only one, while V1920 sample 2 biotypes. Ten biotypes were observed for *L. plantarum* and 6
 373 of them contained only a strain. Also, in this case strains clustere on the basis of their origin. In particular, for
 374 V1975 sample 5 biotypes were found, each of them with a single strain. For V2008 samples 5 clusters were
 375 identified and only one contained a strain (Fig. 6). This association of strains with their origin could be
 376 explained by the existence of metabolic interdependencies between strains. It is possible that, specific strains
 377 may have been selected and be prevalent with respect to the remaining microbial community.



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 379
 380 Fig. 4. Percentage of bacteria species.
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 385 Fig. 5. RAPD-PCR cluster analysis of *G. oxydans* isolates. Unweighted pair group method with arithmetic
 386 mean (UPGMA) dendrogram derived from comparison of the RAPD-PCR patterns of the isolates obtained
 387 with primer M13.
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390

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392 Fig. 6. RAPD-PCR cluster analysis of *L. plantarum* isolates. Unweighted pair group method with arithmetic
 393 mean (UPGMA) dendrogram derived from comparison of the RAPD-PCR patterns of the isolates obtained
 394 with primer M13.

395

396 3.4. Organic acids content

397 Organic acids are produced through hydrolysis, biochemical metabolism and microbial actions during the
 398 fermentation process. Citric, tartaric, malic, succinic, lactic and acetic acids were detected in all samples
 399 (Table 1). Malic and succinic acids were the main ones with values ranging from 8.49 g/L (V1975) to 11.76
 400 g/L (V2008) and from 4.15 g/L (V1895) to 7.73 g/L (V2008), respectively. Citric acid was present at low
 401 concentrations around 0.3 g/L in all samples, while tartaric, and acetic acids had values of about 2 g/L.
 402 Lactic acid was detected only in small amounts. Organic acids have different origins, mainly grapes and
 403 microbial metabolism. The presence of succinic acid is related to yeast metabolism and the high
 404 concentration detected is in agreement with cell counts. Acetic acid and lactic acid are mainly produced
 405 during alcohol and malolactic fermentations, and small amounts are also produced during thermal treatment
 406 of must due to sugar degradation (Xia, Zhang, Duan, Zhang, & Wang, 2020). Malic, citric, and tartaric acids
 407 mainly derive from grapes (Xia et al., 2020). They could all react with sugars, through a condensation
 408 reaction, followed by CO₂ production, to give brown compounds (Lewis & Quackenbush, 1949). The same
 409 organic acids were detected also in *Vino cotto* samples by other authors with similar amounts (Piva et al.,
 410 2008; Di Mattia, Sacchetti, Seghetti, Piva, & Mastrocola, 2007) as well as in products obtained in a similar
 411 way such as vinegars. Similar amounts of malic, acetic, and citric acids were detected in botrytized wines
 412 which are characterized by high sugar concentration (Kiss & Sass-Kiss, 2005).

413

414 3.5. Determination of volatile profile

415 The volatile metabolites are shown in Table 2. Aroma compounds belonged to seven different families:
 416 esters, higher alcohols, aldehydes, alkanes, and acetals. Esters and higher alcohols were the main compounds
 417 detected.

418 Esters showed total amounts ranging from 32.80 mg/L (V1895) to 96.17 mg/L (V1920). Ethyl esters of fatty
419 acids were the most represented group. Their concentration depends on several factors including yeast
420 species, fermentation temperature, aeration degree, and sugar content (Schreier, 1980) and have very
421 pleasant fruits, honey, and sweet scents which contribute to the aromatic finesse of wines (Ugliano &
422 Henschke, 2009). The main esters were butanedioic acid, diethyl ester (diethyl succinate) and ethyl acetate
423 with values varying from 4.63 mg/L (V1980) to 35.64 mg/L (V1920) and from 8.69 mg/L (V2008) to 13.78
424 mg/L (V1920), respectively. Butanedioic acid, diethyl ester is formed through the esterification of succinic
425 acid and is associated to fruity melon and cooked apple notes. This compound occurs naturally in apples,
426 grapes, and cocoa and its odor threshold has been set at 1.2 mg/L (Peinado, Moreno, Bueno, Moreno, &
427 Mauricio, 2004). Its concentration increases during wine storage and aging (Cortés-Diéguez, Rodríguez-
428 Solana, Domínguez, & Díaz, 2015). Butanedioic acid, diethyl ester was also observed to increase in sweet
429 wines such as Sherry type wines (Moreno-García, Raposo, & Moreno, 2013) and natural sweet wines
430 (Issa-Issa et al., 2019). Ethyl acetate is formed by the action of yeasts during fermentation, and by the action
431 of AAB during ageing (Nogueira & Nascimento, 1999). Ethyl acetate is perceived as the odour of nail
432 polish remover when occurs above the sensory threshold (12 mg/L); at low amounts it confers fruity aroma
433 properties and adds complexity to wine.

434 Higher alcohols represent the other important group of volatiles identified in wine. The group is composed of
435 aliphatic and aromatic alcohols, most of which are products of yeast fermentation (Kotseridis & Baumes,
436 2000). Their concentrations ranged from 17.96 mg/L (V1890) to 49.47 mg/L (V1920) and the main
437 compounds detected were 1-butanol, 3-methyl and phenylethyl alcohol with values varying from 7.77 mg/L
438 (V1890) to 27.63 mg/L (V1920) and from 4.49 mg/L (V2008) to 12.11 mg/L (V1920), respectively. These
439 higher alcohols are positively related to the vegetal/pepper note of aged red wines and negatively related to
440 the intensity of the toasted and woody-old attributes (Aznar, López, Cacho, & Ferreira, 2003). Recently
441 have been also proved the sensory importance of the pair 1-butanol, 3-methyl and isoamyl alcohol on wine
442 aroma perception and that the effects of the alcohols are extremely dependent on the aromatic context. In
443 poor aromatic contexts, lacking of specific aroma nuances other than those of the wine aroma base, the
444 sensory effects are negligible confirming the aroma buffering effects of such wine aroma base. Only in
445 contexts in which aroma notes are clearly perceived, the effects become clearly noticeable (De-la-Fuente-
446 Blanco, Sáenz-Navajas, & Ferreira, 2016).

447 Furfural and its derivatives were the main aldehydes detected. Their occurrence in “mothers” of *Vino cotto* is
448 probably related to the production process. In fact, they are considered as some of the main products of the
449 browning process (and markers of a heat treatment); and thus, they can play a role in delivering a dried and
450 cooked fruit flavor (Loizzo et al., 2013).

451 Among acetals 1,3-dioxolane, 2,4,5-trimethyl was the main compound detected and its occurrence has been
452 already described in “Zaoheibao” wine (Duan, Liu, Lv, Wu, & Wang, 2020).

453 Alkanes were well represented too, probably due to the coking process of the must. 3,3'-bi-p-menthane was
454 the most abundant with values ranging from 15.78 mg/L (V1980) to 29.42 mg/L (V1890). It is associated to

455 fennel and peppermint odor (Shigeto, Wada, & Kumazawa, 2020), and thus, could contribute to the balsamic
456 notes of Vino cotto.

457

458 Table 1 Organic acids detected in “mothers” of Vino cotto. Data are expressed as g/L. $p < 0.01$.

459

Samples	Citric acid	Tartaric acid	Malic acid	Succinic acid	Lactic acid	Acetic acid
V1890	0.38 ± 0.03	1.69 ± 0.08	9.82 ± 0.31	6.54 ± 0.23	0.09 ± 0.02	2.26 ± 0.03
V1895	0.29 ± 0.04	1.88 ± 0.04	11.83 ± 0.45	4.15 ± 0.08	0.79 ± 0.06	2.97 ± 0.12
V1920	0.45 ± 0.05	1.03 ± 0.12	9.63 ± 0.62	7.41 ± 0.21	0.53 ± 0.11	2.99 ± 1.12
V1975	0.37 ± 0.02	1.65 ± 0.05	8.49 ± 0.43	5.72 ± 0.62	0.04 ± 0.01	2.41 ± 0.29
V2008	0.44 ± 0.04	1.97 ± 0.05	11.76 ± 0.67	7.73 ± 0.34	0.12 ± 0.03	2.54 ± 0.23

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487 Table 2 Volatile compounds found in tested samples expressed as mg/L. p < 0.01.

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Compounds	Sample				
	V1990	V1895	V1920	V1980	V2008
Esters					
10-Bromodecanoic acid, ethyl ester			0.27 ± 0.01	0.12 ± 0.03	
1-Butanol, 2-methyl-, acetate	0.12 ± 0.02	0.19 ± 0.01	0.20 ± 0.02		0.30 ± 0.04
1-Butanol, 2-methyl-, acetate	0.61 ± 0.03	1.29 ± 0.21	1.70 ± 0.11	0.66 ± 0.05	1.41 ± 0.04
2,5-Dichloro-propionic acid, ethyl ester		0.11 ± 0.01			
2-Butenoic acid, 2-methyl-, 2-phenylethyl ester					
2-Propenoic acid, 1-methylbutyl ester			0.27 ± 0.05		
2-Butyl-4-nitro-pent-4-enoic acid, methyl ester					
Acetic acid, 2-phenylethyl ester	0.41 ± 0.04			0.47 ± 0.03	0.40 ± 0.07
Acetic acid, hydroxy-, ethyl ester					
Benzoic acid, ethyl ester	0.52 ± 0.05	0.18 ± 0.03			0.29 ± 0.04
Butanedioic acid, diethyl ester	12.66 ± 0.75	13.74 ± 0.43	35.64 ± 0.56	4.63 ± 0.12	12.96 ± 0.28
Butanedioic acid, ethyl 3-methylbutyl ester					0.14 ± 0.01
Butanedioic acid, hydroxy-, diethyl ester, (-)-					
Butanoic acid, 2-methyl-, ethyl ester	0.07 ± 0.03	0.16 ± 0.02			
Butanoic acid, ethyl ester	0.05 ± 0.02	0.16 ± 0.02	0.26 ± 0.02	0.13 ± 0.04	0.13 ± 0.02
Decanoic acid, ethyl ester			14.82 ± 0.27	5.57 ± 0.26	1.01 ± 0.05
Dodecanoic acid, ethyl ester			3.00 ± 0.06	1.11 ± 0.12	0.16 ± 0.09
E-11-Hexadecanoic acid, ethyl ester					
Ethyl 9-decanoate				0.24 ± 0.02	0.06 ± 0.01
Ethyl Acetate	12.40 ± 0.21	12.39 ± 0.23	13.78 ± 0.32	12.57 ± 0.26	8.69 ± 0.04
Hexadecanoic acid, ethyl ester			1.02 ± 0.02		
Hexanoic acid, 2-methyl-, 1-methylpropyl ester				0.10 ± 0.02	
Hexanoic acid, ethyl ester	1.10 ± 0.12	0.61 ± 0.05	3.77 ± 0.24	1.92 ± 0.06	2.68 ± 0.17
Methyl 2-methylbutanoate				2.15 ± 0.19	0.11 ± 0.02
Methyl 2-methylpentadecanoate					0.07 ± 0.02
Nonanoic acid, ethyl ester				0.15 ± 0.03	
Octanoic acid, ethyl ester	0.90 ± 0.04	0.40 ± 0.02	13.51 ± 0.15	4.50 ± 0.12	1.28 ± 0.15
Peranoic acid, diethyl ester					
Peranoic acid, 2,2-dimethyl-, methyl ester	0.74 ± 0.231	1.30 ± 0.03	3.64 ± 0.05	14.71 ± 0.23	6.90 ± 0.12
Peranoic acid, 2,4-dimethyl-, methyl ester	1.80 ± 0.21				1.91 ± 0.17
Peranoic acid, 2-methyl-, butyl ester			0.21 ± 0.09		
Peranoic acid, 4-oxo-, ethyl ester		0.52 ± 0.03			
Propanoic acid, 2-methyl-, ethyl ester			3.76 ± 0.32		0.29 ± 0.07
Propanoic acid, 3-ethoxy-, ethyl ester		1.57 ± 0.27			
Propanoic acid, ethyl ester		0.17 ± 0.03			
Tetradecanoic acid, ethyl ester					
TOF	39.48	32.80	96.17	49.03	38.90
Higher alcohols					
(S)-3,4-Dimethylpentanol		0.06 ± 0.03	0.72 ± 0.04		
1,3-Butanediol, (S)-			0.66 ± 0.03	0.38 ± 0.05	0.22 ± 0.03
1-Butanol, 2-methyl-	1.60 ± 0.06	3.77 ± 0.11	5.96 ± 0.06	1.70 ± 0.05	1.51 ± 0.06
1-Butanol, 2-methyl-	7.77 ± 0.28	17.68 ± 0.31	27.63 ± 0.39	9.22 ± 0.26	11.30 ± 0.28
1-Propanol, 2-methyl-	0.47 ± 0.02	3.12 ± 0.04	2.10 ± 0.11	0.38 ± 0.03	0.26 ± 0.06
2,2-Dimethyl-1,3-butanediol			0.21 ± 0.02		
2,3-Butanediol	0.20 ± 0.02				
1-[[Trimethylsilyloxy]propoxy]-2-ol					
Phenylethyl Alcohol	7.85 ± 0.21	2.37 ± 0.32	12.11 ± 0.21	7.90 ± 0.21	4.49 ± 0.22
2-Methyl-2-methyl-2-yl-methanol					
TOF	17.96	27.23	49.47	18.68	17.98
Aldehydes					
2-Furancarboxaldehyde, 5-methyl-	1.61 ± 0.4	0.74 ± 0.03			
6-Nonenal, 2,7-dimethyl-	0.09 ± 0.02		3.74 ± 0.06		
2-Furancarboxaldehyde, 5-methyl-					1.25 ± 0.08
5-Hydroxymethylfurfural		0.60 ± 0.04			
Benzaldehyde		1.02 ± 0.05	5.19 ± 0.12	1.23 ± 0.03	1.18 ± 0.03
Pheanal, 2,5-bis(1,1-dimethyl-2-ethyl)-	1.13 ± 0.03			0.71 ± 0.12	
Pheanal, 3,5-bis(1,1-dimethyl-2-ethyl)-		0.09 ± 0.01			
Propenal, 2,3-dihydroxy-, (S)-	0.02 ± 0.01		0.09 ± 0.02	0.04 ± 0.01	0.08 ± 0.02
Pyranic-4-carboxaldehyde, 1-methyl-	0.22 ± 0.05				
TOF	3.07	3.46	9.02	1.96	3.61
Acetals					
1,3-Dioxane, 2,4-dimethyl-			0.10 ± 0.02	0.03 ± 0.01	0.02 ± 0.02
1,3-Dioxane, 2-pentadecyl-					
1,3-Dioxolane, 2,4,5-trimethyl-	4.10 ± 0.23	4.41 ± 0.31	14.68 ± 0.52		0.47 ± 0.03
1,3-Dioxolane, 2-methoxypropyl-2,4,5-trimethyl-		0.29 ± 0.06		1.20 ± 0.09	
1,3-Dioxolane, 4,5-dimethyl-2-pentadecyl-		0.21 ± 0.05			
Pyranose, 1-(1-ethoxyethoxy)-		0.22 ± 0.04			
Pyranose, 1,1-dithoxy-			0.40 ± 0.02		
TOF	4.10	5.22	15.17	1.20	0.48
Alkanes					
3-Prop-2-enoyloxytetradecane	0.01 ± 0.01				
3,2'-Bi-p-menthane	29.42 ± 0.15	26.91 ± 0.21	24.03 ± 0.33	15.79 ± 0.22	18.48 ± 0.26
3-Trifluoroacetyloxytetradecane				0.07 ± 0.04	
4-Trifluoroacetyloxytetradecane				0.20 ± 0.02	0.10 ± 0.03
Butane, 1-(ethoxyoxy)-3-methyl-			0.40 ± 0.03		0.06 ± 0.02
Butane, 1,1-dithoxy-3-methyl-		0.13 ± 0.06			0.06 ± 0.02
Ethane, 1,1-dithoxy-			3.75 ± 0.14		
TOF	29.43	27.04	27.18	16.15	18.71

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493 *3.6. Principal component analysis (PCA)*

494 PCA allowed 73.41% of the total variance to be explained by the first two principal components (Fig. 7).

495 Based on the distribution of samples 3 groups can be identified. V1920 sample appeared far from the others

496 for its composition in terms of citric, succinic and acetic acids, esters, aldehydes, acetals, and higher

497 alcohols. V2008 and V1975 samples clustered together for LAB and yeast counts. V1890 and V1895

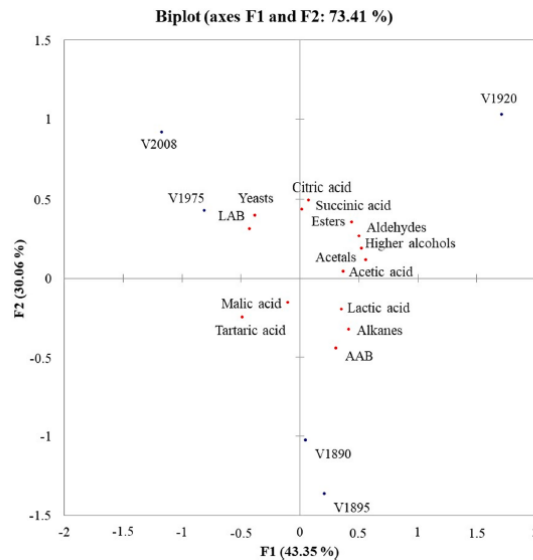
498 belonged to the 3rd group and were differentiated from the others for lactic acid, AAB, organic acids, and

499 alkanes. Obtained data suggested that the aging period exerts a strong effect in the definition of “mothers” of

500 Vino cotto traits indicating that it should be possible to modulate the characteristics of Vino cotto using
501 “mothers” of different years.

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505 Fig. 7. PCA analysis encompassing organic acids, volatile compounds and cell counts.

506

507 4. Conclusions

508 “Mother” of Vino cotto is a highly selective environment. Isolated strains – especially *Starm. bacillaris*, *L.*
509 *plantarum* and *G. oxydans* – showed genetic diversity. Therefore, the source of a strain/species is a key
510 factor that affects the final genetic diversity of the individual population and different origins can favour or
511 disfavour certain species in terms of their strain genetic diversity development. The strict relation between
512 bacteria and origin suggests that they probably play a major role in the definition of final product
513 characteristics. Knowing the resident population present in the barrels can improve the knowledge of this
514 traditional product and could represent a prerequisite for the selection of strains ad hoc for Vino cotto.

515 Further studies on physiological and genetic characteristics of microbiota could contribute to a better
516 understanding of microbial ecology and to improve the quality of this old traditional product maintaining a
517 strong link with the territory.

518

519 Declaration of Competing Interest

520 The authors declare that they have no known competing financial interests or personal relationships that
521 could have appeared to influence the work reported in this paper.

522

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